

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under
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First Named Inventor or
Application Identifier Jan E. Schnitzer

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Title of
Invention IMMUNOISOLATION OF CAVEOLAE

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
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1. ☐ Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 26]
(preferred arrangement set forth below)
 - Descriptive title of the invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to microfiche Appendix
 - Background of the Invention
 - Summary of the Invention
 - Brief Description of the Drawings
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 2]
4. ☒ Oath or Declaration/POA [Total Pages 3]
(Unexecuted)
 - a. ☐ Newly executed (original or copy)
 - b. ☐ Copy from a prior application (37 C.F.R. 1.63(d))
(for continuation/divisional with Box 17 completed)
[NOTE Box 5 below]
 - i. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting
inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy
of the oath or declaration is supplied under Box 4b, is considered
as being part of the disclosure of the accompanying application
and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
 - a. ☐ Computer Readable Copy
 - b. ☐ Paper Copy (identical to computer copy)
 - c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & documents)
9. ☐ 37 C.F.R. 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503) (2)
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14. ☐ Small Entity ☐ Statement filed in prior application,
Statement(s) Status still proper and desired
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(if foreign priority is claimed)
16. ☐ Other:.....

17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: _____/_____

Prior application information: Examiner: _____ Group Art Unit: _____

18. CORRESPONDENCE ADDRESS

NAME	Patricia Granahan, Esq.				
	HAMILTON, BROOK, SMITH & REYNOLDS, P.C.				
ADDRESS	Two Militia Drive				
CITY	Lexington	STATE	MA	ZIP CODE	02421-4799
COUNTRY	USA	TELEPHONE	(781) 861-6240	FAX	(781) 861-9540

Signature	<i>Patricia Granahan</i>	Date	December 8, 1998
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Inventors: Jan E. Schnitzer and Philip Oh

Attorney's Docket No.: BIDMC98-20

IMMUNOISOLATION OF CAVEOLAE

GOVERNMENT SUPPORT

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5 the invention.

BACKGROUND OF THE INVENTION

Cholesterol and glycolipids self-associate in lipid bilayers to form organized compositional microdomains (Thompson, T.E., *et al.*, *Annu. Rev. Biophys. Chem.* 14:361 (1985)). Glycosyl-phosphatidylinositol (GPI)-anchored proteins and other lipid-
10 linked proteins may preferentially partition into glycolipid microdomains that are resistant to nonionic detergent solubilization (Schroeder, R., *et al.*, *Proc. Natl. Acad. Sci. USA* 91:12130 (1994); Brown, D.A. and Rose, J.K., *Cell* 68:533 (1992); Letarte-Murhead, M. *et al.*, *Biochem. J.* 143:51 (1974); Hoessli, D. and Runger-Brandle, E., *Exp. Cell. Res.* 166:239 (1985); Hooper, N.M. and Turner, A.J., *Biochem. J.* 250:865
15 (1968); Sargiacomo, M. *et al.*, *J. Cell. Biol.* 122:789 (1993); Lisanti, M.P. *et al.*, *J. Cell. Biol.* 123:595 (1993)). GPI-anchored proteins appear to be sorted into glycolipid, detergent-resistant "rafts" in the trans-Golgi network for polarized delivery to the cell surface by smooth exocytotic carrier vesicles which are resistant to detergents and also contain caveolin (Brown, D.A. and Rose, J.K., *Cell* 68:533 (1992); Sargiacomo, M. *et*
20 *al.*, *J. Cell. Biol.* 122:789 (1993); Lisanti, M.P. *et al.*, *J. Cell. Biol.* 123:595 (1993);

Brown, D. *et al.*, *Science* 245:1499 (1989); Simons, K. and van Meer, G., *Biochemistry* 27:6197 (1988); Garcia, M. *et al.*, *J. Cell Sci* 104:1281 (1993); Kurzchalia, T.V. *et al.*, *J. Cell Biol.* 118:1003 (1992); Dupree, P. *et al.*, *EMBO J.* 12:1597 (1993); Hannan, L.A. *et al.*, *J. Cell. Biol.* 120:353 (1993)). Caveolae are smooth membrane invaginations that are also resistant to detergent extraction; they exist on the surface of many different cell types, and are especially abundant in endothelium. Caveolae are apparently also rich in glycolipids, cholesterol, and caveolin (Kurzchalia, T.V. *et al.*, *J. Cell Biol.* 118:1003 (1992); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1759 (1995)). Low-density, Triton-insoluble membranes are frequently equated with caveolae (Sargiacomo, M., *et al.*, *J. Cell Biol.* 122:789 (1993); Lisanti, M.P. *et al.*, *J. Cell. Biol.* 123:595 (1993); Chang, W.-J. *et al.*, *J. Cell. Biol.* 126:127 (1994); Lisanti, M.P. *et al.*, *J. Cell. Biol.* 126:111 (1994)), but recent work has shown they have a mixture of detergent-resistant microdomains (Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995)). Characterization of caveolae shows that they are very enriched in caveolin; the glycolipid GM1; the plasmalemmal CA²⁺-dependent adenosine triphosphatase; and the inositol 1,4,5-triphosphate receptor; these four molecules have all been shown by independent means to reside on the cell surface almost exclusively in caveolae (Dupree, P., *et al.*, *EMBO J.* 12:1597 (1993); Parton, R.G., *J. Histochem. Cytochem.* 42:155 (1994); Rothberg, K.G. *et al.*, *Cell* 68:673 (1992); Montessano, R. *et al.*, *Nature* 296:651 (1982); Fujimoto, T., *J. Cell. Biol.* 120:1147-1193 (1993)) and thus represent key markers of caveolae.

Caveolae have been implicated not only in signaling but also in transport via endocytosis, transcytosis, and potocytosis (Montessano, R. *et al.*, *Nature* 296:651 (1982); Schnitzer, J.E., *Trends Cardiovasc. Med.* 3:124 (1993); Oh, P. *et al.*, *J. Cell Biol.* 127:1217 (1994); Schnitzer, J.E. and Oh, P., *J. Biol. Chem.* 269:6072 (1994); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1759-1763 (1995); Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Milici, A.J. *et al.*, *J. Cell Biol.* 105:2604 (1987); Anderson, R.G. W. *et al.*, *Science* 265:410 (1992)). However, there is disagreement as to whether caveolae serve as signaling centers (see Liu, J. *et al.*, *J. Biol. Chem* 272:7211-7222 (1997), Schnitzer, J.E. *et al.*, *Mol. Biol. Cell* 5:75a (1994);

Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1759-1763 (1995); Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); contrast with Stan, R.-V. *et al.*, *Mol. Biol. Cell* 8:595-605 (1997)). The exact physiological composition and functions of caveolae remain undefined.

5 SUMMARY OF THE INVENTION

The present invention is drawn to methods of producing purified caveolae, as well as the purified caveolae produced by the methods, and uses of the purified caveolae. In the methods, immunoisolation of caveolae is performed, using an antibody that is specific for caveolin and that is able to bind oligomerized caveolin found around
10 intact caveolae. Immunoisolation can be performed on a wide variety of starting materials, including cells of interest, such as cultured cells or cells isolated from a tissue; a tissue itself; cell lysate; microsomes derived from cells or from tissue; or a sample of plasma membranes.

In one embodiment, the starting material can be subjected to membrane
15 disruption method and/or a preliminary separation step prior to the immunoisolation. If such a separation step is performed, the separation is based on a physical characteristic of cell membranes (for example, density, size, or phase separation), in order to provide a starting material containing a concentrated amount of plasma membranes. The initial separated fractions (e.g., the lowest density fractions in a density separation) are then
20 collected, and subjected to the immunoisolation method to separate caveolae from other materials in the initial fractions.

During the immunoisolation, a sample of interest (e.g., the starting material, sample of plasma membranes, or initial fractions) that comprises plasma membranes is incubated, preferably for a brief time period (e.g., for less than approximately 2 hours,
25 preferably for approximately one hour or less), with the antibody that is specific for caveolin. Caveolae that are bound to the antibody are then separated from other materials in the sample of interest, thereby producing purified caveolae.

In a preferred embodiment, a sample of plasma membranes from cells of interest is used as the sample of interest. The sample of plasma membranes can be subjected to a membrane disruption method, such as sonication or shearing, to produce disrupted plasma membranes. If desired, the disrupted plasma membranes can then be subjected to separation based on a physical characteristic of the membrane, as described above. The resultant material is then subjected to the immunoisolation method to separate caveolae from other materials in the initial fractions.

The methods of the invention provide simplified, efficient means to produce purified caveolae, while minimizing contamination and avoiding loss of molecules that dissociated from caveolae with time. The caveolae produced by the methods more closely resemble caveolae in their native state; as the methods eliminate extended immunoisolation methods which would otherwise result in significant loss of various caveolar components as well as adsorption of nonspecific proteins or contaminating membranes. The methods can be used to produce purified caveolae from a wide variety of cells or tissues, including not only endothelial cells and tissues, but also other (non-endothelial) cells and tissues, as well as cultured cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic representation of the binding equilibrium over time of monoclonal antibody CAV to caveolin as it exists around caveolae in plasma membranes (i.e., in oligomerized form).

Figure 2 is a graphic representation of the percent of binding of antibodies CAV, 2297 and Z034, as well as polyclonal antiserum pAB, to caveolin as it exists around caveolae in plasma membranes (i.e., in oligomerized form).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to purified caveolae, methods of producing the purified caveolae; and uses for the purified caveolae. As described herein, various techniques to isolate caveolae were compared to determine the effects of the techniques

on the molecular composition of the final isolated caveolae. It was discovered that a brief immuno-affinity isolation procedure, using an antibody that was specific for caveolin and which bound with high affinity to caveolin in its native state as an oligomeric structure, allowed a simplified and quick isolation of caveolae. Caveolae
5 isolated by this procedure retained more caveolae-related proteins than those isolated by other procedures, and thus were more representative of caveolae in their native state than caveolae isolated by other procedures.

As a result of this discovery, methods are now available to quickly and efficiently isolate caveolae. The methods can be carried out on any tissue, cell type, or
10 cellular fraction whose plasma membranes contain the desired component (caveolae). For example, endothelial cells from representative tissues such as vascular, pulmonary, cardiac, cerebral, nephric, hepatic and endocrinous tissue, including the vascular system, lung, heart, liver, kidney, brain, and other organs, can be used. Alternatively, cells from any other type of tissue can be used, including non-endothelial cells. Cultured cells,
15 cells isolated from a tissue, or a cell lysate can also be used, as can a tissue itself, as well as microsomes derived from cells or from a tissue. The tissue, cells, cell lysate, or microsomes from which the caveolae are isolated is referred to herein as the "starting material".

In the methods of the invention, a sample of interest (e.g., "starting material")
20 that comprises plasma membranes is used. If desired, plasma membranes can first be separated from the other components of the tissue, cells, cell lysate, or microsomes used as the starting material, before immunoisolation is performed. A "sample of plasma membranes," as used herein, refers to plasma membranes that have been separated from other components of the starting material. The sample of plasma membranes can
25 obtained from the starting material by any appropriate method, such as silica coating methods (see U.S. patent 5,281,700; Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1759-1763 (1995); Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al.*, *Mol. Biol. Cell* 5:320(a) (1994); or Schnitzer, J.E. *et al.*, *Mol. Biol. Cell* 5:75a (1994)), or density

gradient centrifugation methods (see, e.g., Smart, E.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:10104-10108 (1995); *Biological Membranes: A Practical Approach* (Findlay and Evans, Eds., IRL Press at Oxford University Press, Oxford, England, 1987); *Subcellular Fractionation: A Practical Approach* (Graham and Rickwood, Eds., IRL Press at

5 Oxford University Press, Oxford, England, 1997); *Cell Biology: A Laboratory Handbook* (J. Celis, Ed., 2nd Edition, Acad. Press, Inc., San Diego, CA, USA, 1998), especially "Isolation and Subfractionation of Plasma Membranes to Purify Caveolae Separately from Glycol-phosphatidylinositol-anchored Protein Microdomains" (P. Oh and J.E. Schnitzer, pp. 34-45)). Alternatively, methods that utilize physiological release

10 of caveolae by inducing budding from plasma membranes can be used to obtain the sample of plasma membranes (see, e.g., Schnitzer, J.E. *et al.*, *Science* 274:239-242 (printer's erratum, *Science* 274:1069) (1996)).

If desired, the starting material comprising plasma membranes, or the sample of plasma membranes, can be subjected to a membrane disruption method, to produce a

15 disrupted plasma membrane sample. The membrane disruption method can be sonication, shearing, or another appropriate membrane disruption method (see, e.g., *Biological Membranes: A Practical Approach* (Findlay and Evans, Eds., IRL Press at Oxford University Press, Oxford, England, 1987); *Subcellular Fractionation: A Practical Approach* (Graham and Rickwood, Eds., IRL Press at Oxford University

20 Press, Oxford, England, 1997); *Cell Biology: A Laboratory Handbook* (J. Celis, Ed., 2nd Edition, Acad. Press, Inc., San Diego, CA, USA, 1998)). The starting material, sample of plasma membranes, or disrupted plasma membrane sample, can also be subjected to a separation method based on a physical characteristic of the membranes, such as buoyant density, size, phase separation, to fractionate the sample. In a preferred method, the

25 sample can be subjected to a separation method based on density. Representative separation methods based on density include sucrose density gradient centrifugation, and centrifugation using an Opti-Prep gradient (Gibco, Inc. (Grand Island, New York). If a separation step is performed, it results in the production of fractions of components. Fractions which contain the greater amount of plasma membranes, with a lesser amount

of contaminants, are isolated for further use. For example, separation based on density results in the production of fractions having different densities. Of these fractions, the initial fractions are collected. "Initial fractions," as used herein, refer to the top (i.e., the least dense) several fractions, that constitute approximately 10-25% of the fractions. In a preferred embodiment, the initial fractions constitute approximately 10-20% of the fractions. If the sample is fractionated based on another characteristic of the plasma membranes besides density, the appropriate fractions (i.e., the fractions that comprise the majority of the plasma membranes with minimal contamination from other components) are collected and used as the "initial fractions".

10 The starting material, sample of plasma membranes, disrupted plasma membrane sample, or the initial fractions are subjected to an immunoisolation method to isolate the purified caveolae. The terms, "immunoisolation" and "immunoaffinity isolation," used interchangeably herein, refer to separation of a component of interest (e.g., caveolae) from other materials using an antibody that is specific for (binds preferentially to) the component of interest. In immunoisolation, a sample of interest (i.e., the starting material, sample of plasma membranes, disrupted plasma membrane sample, or the initial fractions) that comprises plasma membranes is incubated with antibody that is specific for the component of interest; the component of interest, which is bound to the antibody that is specific for the component of interest, is then separated (isolated) from materials (components) that are not bound to the antibody. In a preferred embodiment of the invention, the incubation period of the immunoisolation method is a brief time, that is, the incubation is under about two hours, preferably about one hour or less. The brief time frame allows separation of purified caveolae that more closely resemble caveolae in their native state; as described in the Examples below.

25 Extended immunoisolation results in significant loss of various caveolar components, including loss of the proteins eNOS and heterotrimeric G proteins into solution, and increases adsorption of nonspecific proteins and/or contaminating membranes.

In the immunoisolation method, an antibody that is specific for caveolin is used. The antibody that is specific for caveolin must be an antibody that binds preferentially

to oligomeric caveolin, as caveolin is found in an oligomeric state surrounding intact caveolae (Scherer, P.E. *et al.*, *J.Biol. Chem.* 272(46):29337-46 (1997)). In a preferred embodiment, the antibody binds to caveolin in its native state, and also binds to caveolin in its monomeric state (e.g., in a monomeric state such as after denaturation during Western blot analysis). The antibody allows separation of caveolae from other materials in the sample of interest. Caveolae that have been separated from other materials in the sample of interest are referred to herein as "purified caveolae". Purified caveolae can be bound to the antibody that is specific for caveolin, or can, if desired, be separated from the antibody.

Representative immunoisolation methods include isolation using the antibody specific for caveolin, where the antibody is coated on (attached) to a solid phase, such as beads or spheres. In a preferred embodiment, magnetic beads coated with the antibody are used. The materials that are bound to the antibody that is specific for caveolin (i.e., the purified caveolae), are separated from those materials in the sample of interest that are not bound to the antibody. For example, if a solid phase (e.g., magnetic beads) coated with antibodies is used for immunoisolation, the solid phase (e.g., the beads) together with materials bound to the antibodies that are coated with the solid phase (i.e., the purified caveolae), are separated from materials that are not bound to the solid phase. The purified caveolae can be released from the solid phase, if desired, using standard techniques.

The purified caveolae can be used for the identification of molecules and proteins which are involved in intra-, inter- or trans-cellular transport, in order to utilize such molecules and proteins for delivery of an agent (e.g., an antibody, a drug, a diagnostic agent or a gene) for treatment or therapy. Agents which target a particular component of caveolae may be more easily delivered to the cell and, if desired, into the cell, across one side of a cell to the other side (e.g., across a an endothelial cell layer). Thus, these molecules and proteins can be used to transport agents into and across cell membranes, such as into and/or across the endothelium and, as a result, across the endothelial barrier. The purified caveolae can also be used to target the endothelium,

such as for delivery of an agent for treatment or therapy. Agents which target caveolae may also be more easily delivered to the cell and, if desired, into the cell, across one side of a cell to the other side (e.g., across a an endothelial cell layer). Thus, the caveolae can be used to transport agents into and across cell membranes, such as into
 5 and/or across the endothelium and, as a result, across the endothelial barrier. This is of considerable value because of the role the endothelium plays in many tissues of the body as a barrier to the passage of substances across the endothelium and into the underlying tissue. For example, agents (e.g., antibodies, drugs, diagnostic agents, genes) which bind to the caveolae or to particular components (e.g., molecules or
 10 proteins) can be used to target the caveolae. Alternatively, such agents can be used as transport agents, by conjugating another agent (e.g., a drug or a gene) to the agent which targets the caveolae.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings
 15 of all references cited are hereby incorporated herein by reference.

EXAMPLE 1 Materials and Methods

Materials

Antibodies against caveolin (rabbit polyclonal and mouse monoclonal, clones #2297 and 2234, respectively) were purchased from Transduction Labs (Lexington,
 20 KY), or from Zymed (South San Francisco, CA, mouse monoclonal #Z034). M-450 Dynabeads were purchased from Dynal (New Hyde Park, NY). All other reagents and supplies were obtained as described previously (Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:1759-1764 (1995); Schnitzer, J.E. *et*
 25 *al.*, *Science* 274:239-242 (printer's erratum, *Science* 274:1069) (1996)).

Western and Protein Analysis

The proteins of various tissue fractions were solubilized and separated by SDS-PAGE for direct analysis by silver staining or for Western analysis by electrotransfer to nitrocellulose filters followed by immunoblotting using enhanced chemiluminescence
5 autoradiography and densitometric quantification using ImageQuant, as described previously (Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92;1759-1764 (1995); Schnitzer, J.E. *et al.*, *Science* 274:239-242 (printer's
10 erratum, *Science* 274:1069) (1996)). Briefly, nitrocellulose filters from each gel were probed using primary antibody (dilution range from 1:1,000 to 1:5,000 in Blotto) followed by the appropriate HRP-labeled reporter antibodies (diluted 1:1,000). Sample protein concentrations were measured using the micro-BCA method with BSA as a standard.

Purification of Silica-Coated Endothelial Cell Plasma Membranes and Caveolae

The luminal endothelial cell plasma membranes and caveolae were purified
15 directly from rat lung tissue using an *in situ* silica-coating procedure as described previously ((Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92;1759-1764 (1995)). Rat lung tissue was subfractionated to isolate first the
20 luminal endothelial cell plasma membranes, and then to dislodge and isolate caveolae (Schnitzer, J.E. *et al.*, *J. Biol. Chem.* 270:14399-14404 (1995); Schnitzer, J.E. *et al.*, *Science* 269:1435-1439 (1995); Schnitzer, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA* 92;1759-1764 (1995)). The rat lung microvasculature was perfused *in situ* at 10-13°C
25 via the pulmonary artery with a solution of positively charged colloidal silica particles which coated the intimal endothelial cell surface membrane directly in contact with the circulating blood and created a stable silica pellicle that specifically marked this membrane and facilitated its purification from tissue homogenates by centrifugation.

Electron microscopy showed that the sedimented pellets (P) contain silica-coated endothelial cell plasma membranes with many associated caveolae and little detectable contamination from other tissue components. Biochemically, P displayed ample enrichment for various endothelial cell surface markers relative to the starting whole lung homogenate (H) while excluding various other proteins found in intracellular organelles or other cell surfaces. The caveolae attached on the cytoplasmic side of the membranes opposite to the silica coating were stripped from these membranes by shearing during homogenization at 4°C. then, they were subjected to sucrose density centrifugation to yield a low buoyant density fraction of intact caveolar vesicles (V) well separated from the pellet containing resedimented silica-coated membranes stripped of the caveolae (P-V) and enriched in caveolar but not other markers. Alternatively, caveolae were separated from the plasma membranes (P) by sonication and then isolated in a low buoyant density fraction (referred to herein as PC) by sucrose density gradient centrifugation.

15 *ELISA*

The reactivity of caveolin antibodies with intact caveolae on the purified, silica-coated endothelial cell plasma membranes was assessed by ELISA. Briefly, equal aliquots of P (silica-coated luminal endothelial cell plasma membranes, isolated as described above) (5 µg in 100 µl) were placed in each well of a 96-well tray for drying overnight. After washing, the wells were blocked for 1 hour with EWB (2% ovalbumin and 2 mM CaCl₂ in PBS), incubated with EWB alone or EWB containing caveolin antibody, washed, incubated with reporter antibody conjugated to horseradish peroxidase (1:500 in EWB), and washed again. A substrate solution (50 mM Na₂HPO₄, 25 mM citric acid, 1.2 mg/ml of o-phenylenediamine dihydrochloride and 0.03% H₂O₂) was added and the reaction was stopped with 4 M H₂SO₄ before reading the signal using a Molecular Device Thermomax microplate reader.

Immuno-affinity Isolation of Caveolae

Magnetic immunoisolations were performed as follows: M450 DynaBeads conjugated with anti-mouse or -rabbit IgG were washed 3 times with PBS by resuspension and magnetic separation and then incubated for 4-8 hours with the desired antibody (10^7 M450 beads and 25 μ g IgG). After washing 3 times with PBS, the beads were resuspended and incubated for one hour at 4°C with various starting membrane subfractions (SM) before washing and magnetic separation to isolate two fractions: material bound to the beads (BS) and material not bound to the beads (UB). The SM, BS and UB fractions were subjected to SDS-PAGE and Western analysis. All experiments described herein were performed without added BSA as a blocker.

Simplified Purification of Caveolae from Plasma Membranes

Homogenates of tissue or cultured cells were subjected to Percoll gradient centrifugation to isolate the plasmalemmal fraction, in order to provide a sample enriched in plasma membranes. Lung tissue was homogenized in buffer (0.25 M sucrose/1 mM EDTA/20 mM Tricine, pH 7.8) and the lung homogenate was filtered sequentially through 53 and 30 μ m Nytex filters. The cell/filtered tissue homogenates were subjected to centrifugation at 4°C and the supernatant was saved. The resulting pellet was resuspended in 3 ml of buffer and subjected again to homogenization and centrifugation as above. The two supernatants were then combined and mixed with 30 ml of 30% Percoll in buffer. After centrifugation with a SW28 rotor at 84,000xg for 45 minutes at 4°C (no brakes), a single membranous band was collected. The band was readily visible about 2/3 from the bottom of the tube. The band was pelleted by diluting the suspension 2-3 fold with MBS before centrifugation at 15,000xg at 4°C for 2 hours. This band, referred to herein as the PM (plasma membrane) fraction, is enriched in plasma membranes.

To isolate the caveolae, the PM fraction was resuspended in 1 ml Mes-buffered saline (MBS) and sonicated on "high" (2 x 10 seconds; Branson sonicator), followed by immediate cooling on ice. The sonication procedure was repeated 3 times. The

sonicated membranes were mixed with 50% Opti-Prep to yield a final concentration of 23% in a 3 ml total volume. After adding a continuous layer of 20-10% Opti-Prep to the SW 55 tube, the suspension was spun at 23,500xg for 90 minutes at 4°C in a SW 55 rotor. Depending on the amount of PM loaded, 5-7 distinct membranous bands were visible. The first 2 bands (the “initial fraction”), or the top fourth of the tube, were then collected, and the membranes were pelleted by dilution and centrifugation as described above.

For immunoisolation of the caveolae, this membrane pellet was resuspended in 100 µl MBS for incubation with anti-mouse IgG Dynabeads (2×10^7 beads) prebound with 25 µg of CAV and processed as described above.

EXAMPLE 2 Characterization of an Antibody Reactive with the Caveolin Cage of Caveolae

Monoclonal antibody clone 2234(Transduction Labs, Lexington, KY), referred to herein as CAV, is not only specific for caveolin, but also binds caveolin in its native state as an oligomeric structural cave surrounding intact caveolae. Previous work (Scherer, P.E. *et al.*, *J. Biol. Chem.* 270:16395-16401 (1995)) indicated that this antibody specifically binds the α -isoform of caveolin-1 via a specific epitope found in the N-terminal segment that is not present in the β -isoform.

ELISA analysis, as described above, revealed that CAV was able to bind caveolin expressed on the purified silica-coated endothelial cell membranes (P); in addition, kinetic and binding analysis indicated rapid and high affinity binding of CAV to the caveolae in P. Titration of antibody concentration demonstrated that binding was saturable, with maximum binding occurring at about 10 nm. Time course studies revealed that binding equilibrium was achieved rapidly in just 1 hour (Figure 1). Other monoclonal antibodies (2297 and Z034), also specific for caveolin by Western analysis, showed very little to no binding to caveolin in P by ELISA (Figure 2). Polyclonal antibody showed a little reactivity, but only at higher concentrations and at levels insufficient to be useful for immunoisolation (Figure 2).

The ability of CAV to react with fraction V (the low buoyant density fraction containing caveolae, isolated from P as described above) was then examined by performing immuno-affinity isolation of the caveolae as described above. Results indicated that CAV bound to intact caveolae in V, to allow immunoisolation. Greater than 95% of the caveolin in V was bound to the CAV-coated beads in the bound (BD) fraction, with little remaining behind in the unbound (UB) fraction. Furthermore, nearly all of the proteins detected in V fractionated to BD, rather than UB, upon CAV immunoisolation of the caveolae.

In contrast, other caveolin antibodies (2297, Z034) were ineffective, with nearly all of the caveolin and protein signal detected in UB rather than BD. The polyclonal antibody was poor, but partially effective. CAV thus appeared to be a high affinity antibody reacting quite rapidly and specifically with caveolin not only in its monomeric state after denaturation during Western analysis, but also in its native oligomeric state surrounding intact caveolae found in P and V.

EXAMPLE 3 Comparison of Sonication and Shearing for Isolation of Low Density Membranes

A comparison of the efficacy of sonication and of shearing in removing and isolating caveolae from the silica-coated cell plasma membranes (P) was performed, as described above. Sonication and shearing were both quite effective in not only removing caveolae from P (>80% loss in caveolin signal in the resedimented silica-coated membranes), but also yielding a caveolin-enriched, low density vesicular fraction after continuous sucrose density centrifugation. Western blot analysis indicated that, relative to P, the V fraction (obtained by shearing) and the PC fraction (obtained by sonication) were both quite enriched in caveolin by more than 10-fold. Although PC and V had many proteins in common (eNOS, G proteins, annexin II and PKC), many other proteins found in P were readily detected in PC but not V, including the cytoskeletal protein β -actin, the GPI-anchored protein urokinase-plasminogen activator receptor (uPAR) and angiotensin converting enzyme (ACE). Neither ϵ -COP (markedly

depleted in P) nor the GPI-anchored 5'-nucleotidase (5'NT) (enriched in P) were apparent in V or PC. Thus, V was quite distinct in molecular composition from P and PC.

SDS-PAGE gels further confirmed the differences between the fractions: V had the simplest profile, with the fewest detectable proteins, whereas PC had many more protein bands than V but less than that seen in P. Although PC contained many, if not all, of the proteins apparent in V, it also contained many proteins found in P but not in V. This analysis indicated that sonication and shearing of P yielded vesicular fractions with the same low buoyant density but quite different molecular composition. It appeared that sonication of P released additional low density vesicles not found in V.

EXAMPLE 4 Immunoisolation from PC and V with CAV Antibody

The PC and V fractions described above were subjected to identical immunoaffinity isolation, as described above, for one hour using CAV bound to magnetic beads. The starting material (referred to as "SM"; SM here was either PC or V), the material not bound to the immuno-magnetic beads (UB) and the material bound to the beads (BD) were examined by Western blot analysis. The fractions were analyzed first to assess the relative distribution of the molecules by using equivalent volume conditions (the final volumes were equal in each fraction and the same volume of each fraction was added to the gel lanes, so that the same percentage or proportion of each fraction was analyzed).

Although ample signals for each of the assessed molecules were seen in the starting material SM (PC), only a subset of these molecules were found in BD, namely, the vesicles bound to the caveolin-antibody beads. Caveolin gave the strongest signal in BD relative to both UB and SM. PKC α and the G protein G $_{\alpha q}$, as well as to a lesser extent eNOS and G $_{\alpha s}$, were detected in BD, yet significant levels of each remained behind in UB. Little to no signal for annexin II, β -actin and uPAR was detected in BD; these proteins remained nearly completely in UB.

The results where fraction V was used as the starting material [SM (V)] differed significantly. In each case where a signal was present in SM (V), little to no signal was found in UB and nearly all of the signal was recovered in BD. For example, more than 95% of the signal in SM (V) for caveolin, G proteins, eNOS and PCK α was found in BD. uPAR and β -actin were not detected in any of these fractions. Thus, nearly all of the caveolin was accessible and able to interact with the immuno-beads, resulting in quantitative isolation of nearly all of the starting material. This is in contrast with the results when PC was used as the starting material, where a significant amount of caveolin was inaccessible to immunoisolation.

To be certain that proteins in PC, for which a mild to nil signal was detected in BD, were not simply diluted out by maintaining equivalent volume conditions during the analysis, equal amounts of protein from each fraction were loaded onto the gels before Western analysis. This resulted in a signal for eNOS, G protein and annexin II that was more readily detected in BD from PC as the starting material. By contrast, little to no β -actin or uPAR was again detected in BD, with ample signal present in UB, consistent with these molecules not normally residing concentrated in caveolae. Analysis of fractions under volume vs. protein equivalence gave noticeably different results, revealing readily apparent differences between PC and V, as well as striking similarities between V and the immunobound fractions (UB) from both PC and V.

EXAMPLE 5 Immunopurification of Caveolae from Plasma Membrane Isolated without Silica Coating

Rat lungs flushed free of blood were processed to isolate a plasma membrane fraction (PM) using Percoll gradient centrifugation, and then a caveolin-enriched fraction (AC) was isolated by sonication of PM followed by sucrose density centrifugation, as described by Smart *et al.* (Smart, E.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 92:10104-10108 (1995)). Western analysis was performed, revealing that PM was enriched in various plasmalemmal marker proteins including caveolin, 5'NT, β -actin, eNOS, and uPAR. PM also contained significant levels of ϵ -COP, indicating the

presence of contaminating Golgi and endosomal membranes. When PM was subfractionated as described by Smart *et al.* (supra) by sonication to produce small vesicles that were isolated by centrifugation, first as a broad band on a continuous sucrose gradient for separation, and then again on a step gradient effectively to
 5 concentrate the low density buoyant vesicles, the resulting AC fraction was quite enriched in caveolin and eNOS (>5-fold) which is consistent with past reports (Shaul, P. *et al.*, *J. Biol. Chem.* 271:6518-6522 (1996)). It was also mildly enriched in β -actin and the GPI-anchored proteins 5'NT and uPAR, again consistent with past studies (Smart *et al.*, *supra*). Although not enriched, molecules such as annexin II as well as ϵ -COP were
 10 easily detected in the AC fraction. The proteins detected in silver-stained gels for AC and PM were similar but clearly not identical.

AC was also subjected to immunoisolation using the CAV antibody, as described above. Western analysis of the starting material SM (AC), as well as the UB and BD fractions, under equal protein conditions indicated that many proteins originally
 15 detected in AC did exist in the immunoisolated caveolin-coated vesicles (i.e., the caveolae), whereas many other proteins in starting material AC were not constituents of these vesicles. Caveolin in SM (AC) was found enriched in BD relative to UB. eNOS was clearly detected in BD with little signal in UB. Annexin II partitioned approximately equally between SM (AC), UB and BD. The GPI-anchored proteins
 20 5'NT and uPAR, as well as the Golgi/endosomal marker ϵ -COP and the cytoskeletal protein β -actin were not readily detected in BD, but rather, remained in UB. To be certain that the GPI-anchored proteins did not dissociate from membranes into solution, the UB fraction was subjected to centrifugation. It was found that the GPI-anchored proteins were in the membrane pellets. Thus, CAV immunoisolation purified the
 25 caveolin-coated caveolae in the AC fraction away from other membrane microdomains, including those rich in GPI-anchored proteins and/or cytoskeletal proteins.

EXAMPLE 6 CAV Immunoisolates Nearly All Proteins and Caveolae in V, but not PC or AC

The low density, caveolin-rich vesicles isolated from each fractionation procedure (V, PC and AC), were subjected identically to immunoisolation for one hour using CAV before performing SDS-PAGE as well as Western analysis for caveolin on the immuno-separated fractions. The gels revealed that nearly all of the membranes in V bound to the CAV beads, and that nearly all of the proteins as well as caveolin in V fractionated with the immuno-beads in BD, with little to none remaining in the UB fractions. Except for some bands coming from the IgG heavy and light chains, and BSA included by the manufacturer with the beads, the protein profile of V in SM was identical to BD. Conversely, when CAV was replaced with a clathrin antibody as a control, little to none of the proteins and caveolin originally in SM (V) was detected in BD, but remained in UB.

For PC and AC, the results were strikingly different. Proteins were readily detected in both the BD and UB fractions, consistent with the presence of a significant population of low density vesicles that were not reactive with the caveolin antibody. Increasing the amount of antibody and immuno-beads several fold yielded the same result. The protein profiles in BD and UB were quite different, yet the proteins detected in BD from PC and AC were very similar to each other, as well as to those seen in V (also BD from V). Thus, subjecting the AC and PC fractions to immunoisolation revealed the existence of a heterogeneous population of distinct vesicles rather than a homogeneous population of caveolin-coated vesicles.

This analysis was in concordance with the Western analysis (above), that showed that many molecules in SM (PC) or SM (AC) were split between UB and BD, whereas for V, all molecules in SM (V) fractionated to BD with little to none remaining in UB. The same proteins detected in V (before immunoisolation as well as after) were found in the immunoisolated caveolae from PC and AC. These results indicated that V, but not AC nor PC, contained a homogeneous population of purified caveolin-coated caveolae. For PC and for AC, immunoisolation was a necessary additional step to

purify caveolae. The caveolae immuno-purified from PC and AC appeared to be biochemically identical to each other as well as to those of V.

EXAMPLE 7 Time-dependent Translocation of eNOS into Solution

Overnight immuno-isolations, and 1 hour immunoisolations, were performed as described above in Example 6, to assess the ability of CAV antibody to isolate caveolae. Immunoblots of immunoseparated fractions indicated that although caveolin was almost completely detected in BD for both the 1 hour and overnight incubations, eNOS and G_{as} were in BD in the 1 hour incubation, but largely absent from BD after the overnight incubation. Although it was clear that eNOS and G_{as} were initially a part of the floating low density vesicles in V, the overnight incubation caused a substantial release of these signaling molecules from the caveolin-coated caveolae into solution. This apparent dissociation was confirmed in experiments performed in the absence of antibody, where equal aliquots of P were placed in solution for times ranging from minutes to 16 hours, before sedimenting the membranes at 100,000xg and processing the nonparticulate supernatants and membrane pellets for Western analysis. As early as 2 to 4 hours, a loss in eNOS and G_{as}, but not caveolin, was detected from the pelleted membranes, with a concomitant gain in the supernatant. At 1 hour or less, all of the signal detected for these three proteins was detected in the membrane pellet. Thus, with time, these lipid-anchored signaling molecules dissociated from the membrane into solution.

20 EXAMPLE 8 Simplified Immunopurification of Caveolae From Plasma Membranes

A simplified approach to purifying caveolae from cells and tissues was developed. First, a plasma membrane-enriched fraction, equivalent to PM described above, was isolated from tissue and/or cell homogenates by standard centrifugation techniques (e.g., using Percoll gradients). This PM was subjected to vigorous sonication, and then loaded onto a continuous Opti-Prep gradient for centrifugation at 52,000xg for 90 min. The top 25% of the gradient (first 4 fractions) was collected. This caveolin-rich fraction, which contained much less ϵ -COP and GPI-anchored proteins

than vesicles floating at higher densities, was then subjected to immunoisolation using CAV antibody.

This procedure yielded purified caveolin-coated caveolae equivalent in molecular composition to those derived by CAV immunoisolation of PC, V, and AC.

- 5 This procedure shortened the procedure by avoiding the additional Opti-Prep centrifugation step as performed in the AC isolation.

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the

- 10 scope of the claims.

CLAIMS

What is claimed is:

1. A method of producing purified caveolae, comprising the step of subjecting a sample of interest comprising plasma membranes to an immunoisolation method
5 to separate caveolae from other materials in the sample of interest, wherein the immunoisolation method comprises incubating the sample of interest with an antibody that is specific for caveolin and which binds to oligomerized caveolin, and separating caveolae that are bound to the antibody from other materials in the sample of interest, thereby producing purified caveolae.
- 10 2. The method of Claim 1, wherein the sample of interest is selected from the group consisting of: cultured cells, cells isolated from a tissue, cell lysate, tissue, and microsomes derived from cells or from a tissue.
3. The method of Claim 1, wherein the sample of interest is a sample of plasma membranes.
- 15 4. The method of Claim 1, wherein the sample of interest is a disrupted plasma membrane sample.
5. The method of Claim 1, wherein the sample of interest is initial fractions of starting material that has been subjected to a separation method based on density.
- 20 6. The method of Claim 1, wherein the antibody that is specific for caveolin is attached to a solid phase.

7. The method of Claim 6, wherein the solid phase is magnetic beads.
8. The method of Claim 1, wherein the immunoisolation method comprises incubating the sample of interest with an antibody that is specific for caveolin for a time period that is less than approximately 2 hours,
- 5 9. The method of Claim 8, wherein the immunoisolation method comprises incubating the sample of interest with an antibody that is specific for caveolin for a time period that is equal to or less than approximately one hour.
10. Purified caveolae prepared by the method of Claim 1.
11. A method of producing purified caveolae, comprising the steps of:
 - 10 a) providing a sample of interest comprising plasma membranes;
 - b) subjecting the sample of interest to a membrane disruption method, thereby producing a disrupted plasma membrane sample;
 - c) subjecting the disrupted plasma membrane sample to an immunoisolation method to separate caveolae from other materials in the
15 disrupted plasma membrane sample, wherein the immunoisolation method comprises incubating the initial fractions with an antibody that is specific for caveolin and which binds to oligomerized caveolin, and separating caveolae that are bound to the antibody from other materials in the disrupted plasma membrane sample,
20 thereby producing purified caveolae.
12. The method of Claim 11, wherein the membrane disruption method of step (b) is shearing.

13. The method of Claim 11, wherein the membrane disruption method of step (b) is sonication.
14. The method of Claim 11, wherein the antibody that is specific for caveolin is attached to a solid phase.
- 5 15. The method of Claim 14, wherein the solid phase is magnetic beads.
16. The method of Claim 11, wherein the immunoisolation method comprises incubating the disrupted plasma membrane sample with an antibody that is specific for caveolin for a time period that is less than approximately 2 hours.
- 10 17. The method of Claim 16, wherein the immunoisolation method comprises incubating the disrupted plasma membrane sample with an antibody that is specific for caveolin for a time period that is equal to or less than approximately one hour.
18. Purified caveolae prepared by the method of Claim 11.
19. A method of producing purified caveolae, comprising the steps of:
- 15 a) providing a sample of interest comprising plasma membranes;
- b) subjecting the sample of interest to a membrane disruption method, thereby producing a disrupted plasma membrane sample;
- c) subjecting the disrupted plasma membrane sample to a separation method based on density, thereby producing fractions of the disrupted plasma membrane sample, and collecting initial fractions of the disrupted plasma membrane sample;
- 20 d) subjecting the initial fractions of the disrupted plasma membrane sample to an immunoisolation method to separate caveolae from the initial

fractions, wherein the immunoisolation method comprises incubating the initial fractions an antibody that is specific for caveolin and which binds to oligomerized caveolin, and separating caveolae that are bound to the antibody from other materials in the initial fractions,

5 thereby producing purified caveolae.

20. The method of Claim 19, wherein the separation method based on density of step (c) is sucrose density gradient centrifugation.

21. The method of Claim 19, wherein the immunoisolation method comprises incubating the initial fractions with an antibody that is specific for caveolin for a
10 time period that is less than approximately 2 hours.

22. The method of Claim 21, wherein the immunoisolation method comprises incubating the initial fractions with an antibody that is specific for caveolin for a time period that is equal to or less than approximately one hour.

23. Purified caveolae prepared by the method of Claim 19.

15 24. A method of producing purified caveolae, comprising the steps of:

- a) providing a sample of plasma membranes from cells of interest;
- b) subjecting the sample of plasma membranes to a membrane disruption method, thereby producing a disrupted plasma membrane sample;
- c) subjecting the disrupted plasma membrane sample to a separation
20 method based on density, thereby producing fractions of the disrupted plasma membrane sample, and collecting initial fractions of the disrupted plasma membrane sample;
- d) subjecting the initial fractions of the disrupted plasma membrane sample to an immunoisolation method to separate caveolae from the initial

fractions, wherein the immunoisolation method comprises incubating the initial fractions an antibody that is specific for caveolin and which binds to oligomerized caveolin, for a time period that is less than approximately 2 hours, and separating caveolae that are bound to the antibody from other materials in the initial fractions, thereby producing purified caveolae.

25. The method of Claim 24, wherein the immunoisolation method comprises incubating the initial fractions with an antibody that is specific for caveolin for a time period that is equal to or less than approximately one hour.

10 26. Purified caveolae prepared by the method of Claim 24.

SECRET

IMMUNOISOLATION OF CAVEOLAE

ABSTRACT OF THE DISCLOSURE

Method of producing purified caveolae are described, in which a sample of interest comprising plasma membranes is subjected to an immunoisolation method in which the sample of interest is incubated with an antibody that is specific for caveolin and which binds to caveolin in its native (oligomeric) state, preferably for a brief time period, and caveolae that are bound to the antibody are separated from other materials in the sample of interest. Purified caveolae produced by the methods, and uses for the purified caveolae, are also described.

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Time (min)	Optical Density (Arbitrary Units)
0	0.000
10	0.030
20	0.055
30	0.085
60	0.110
120	0.115
240	0.115

Figure 1

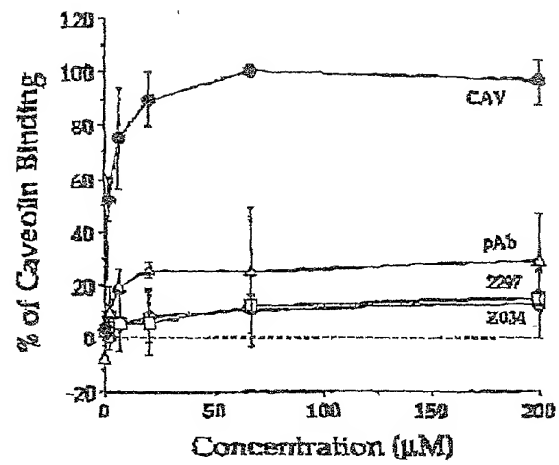


Figure 2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Declaration for Patent Application

As a named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name;

I believe I am the original, first and sole inventor (if only one name is listed) or an original, first and joint inventor (if plural names are listed in the signatory page(s) commencing at page 3 hereof) of the subject matter which is claimed and for which a patent is sought on the invention entitled

IMMUNOISOLATION OF CAVEOLAE

the specification of which (check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application

Number or PCT International Application No. _____

and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

<u>Prior Foreign Application(s)</u>			Priority Not Claimed	Certified Copy Filed?	
				YES	NO
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]	[]	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]	[]	[]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year filed)	[]	[]	[]

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

_____ (Application Number)	_____ (Filing Date)
_____ (Application Number)	_____ (Filing Date)

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known by me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

_____ (Application Serial No.)	_____ (Filing date)	_____ (Status: patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing date)	_____ (Status: patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing date)	_____ (Status: patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing date)	_____ (Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the attorneys and/or agents associated with **Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, Massachusetts 02421-4799, Customer No. 21005,**

[] and _____,

to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Please send correspondence to: [] Customer No. Noted Above OR

[X] Address below:

Patricia Granahan, Esq.
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.
Two Militia Drive
Lexington, MA 02421-4799

Direct telephone calls to: Patricia Granahan, Esq. Telephone No.: 781-861-6240

Direct facsimiles to: Patricia Granahan, Esq. Facsimile No.: 781-861-9540

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole

or first inventor Jan E. Schnitzer

Inventor's Signature _____ Date _____

Residence 1475 Trabert Ransch Road

Encinitas, CA 92024

Citizenship U.S.A.

Post Office Address same as above

1 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2808 2809 2810 2811 2812 2813 2814 2815 2816 2817 28

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